Clinical, genetic and confounding factors determine the dynamics of the in vitro response/non response to clopidogrel

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Introduction
Detrimental platelet activation is a hallmark feature of adverse cardiovascular events. Consequently, antiplatelet therapy became a key therapeutic option for secondary prevention of ischaemic events and consequences thereof (1, 2). Dual antiplatelet therapy with aspirin and clopidogrel is recommended for patients with acute coronary syndromes who are treated medically or undergoing percutaneous coronary intervention (PCI) with stent implantation. Recent studies identified numerous influencing factors for the antiplatelet effect of clopidogrel. Besides genetic predispositions, diverse clinical conditions as well as pharmacological interactions were shown to significantly impair clopidogrel-mediated platelet inhibition. Consequently, these influencing factors may affect clinical outcome after PCI and it is therefore desirable to identify cofounders of HRPR by platelet reactivity testing. It is apparent, that not all assays are sensitive to the same variables, and only cofounders of HRPR that are repeatedly identified by more than one test system may be clinically meaningful. However, treatment adjustment based on platelet function testing has not been associated with improved patients’ outcome. This summary shall provide an overview over current knowledge on influencing factors for clopidogrel-mediated platelet inhibition and aid guidance for critical interpretation of in vitro obtained data on HRPR.

Keywords
Clopidogrel, platelet function testing, influencing factors

Correlation of different test systems
The most frequently used test systems to assess platelet response to clopidogrel treatment were light transmission aggregometry (LTA), multiple electrode platelet aggregometry (MEA), the Ver...
Selection of patients’ cohorts that may influence data

There is no standardised protocol for the enrolment of patients. Reports are on ST-segment elevation myocardial infarction (STEMI) or non-STEMI patients, acute or stable patients, and all of these conditions were evaluated at different time-points of patients’ presentation. Further, patients were either already on maintenance dosage of clopidogrel or newly treated prior to PCI with different loading dosages (8). Maximal inhibition of P2Y12 is achieved 4–5 days after daily intake of 75 mg clopidogrel. This interval can be reduced to 3–5 hours (h) by the administration of a loading dose of 300–600 mg clopidogrel (32). While it was shown that platelet inhibition is less immediately after PCI than 24 h thereafter (33), others did not see a difference between 2 h after loading and several later time points (34). Further, previous studies showed that the extent of pretreatment platelet activation predicts the response to antiplatelet therapy (5, 35). Consequently, patients with exceedingly high pre-treatment reactivity values may be least protected by antiplatelet therapy. As different treatment regimens were used data cannot be compared easily to each other, as they all are novel by themselves. Still, the message conceived is applicable to the entire cohort of patients with atherosclerosis.

Definition of HRPR

A major challenge is how to define HRPR (36). Traditionally, patients’ data are compared to those from a matched healthy control group. This approach is not suitable as atherosclerosis prevails in elder individuals, and age-matched controls may have atherosclerosis themselves, even without any clinical evidence of disease. Further, the biology of platelets in healthy individuals may differ substantially from that in patients. Defining HRPR within a cohort, based on the entire cohorts’ range of platelet reactivity is another option. Thereby, investigators used the upper quartile or quintile of their data to define cut-off values for HRPR. This approach is meaningful within a defined cohort, but possibly does not allow comparisons between cohorts. The probably best measure is clinical outcome, defining cut-off levels based on receiver-operating characteristic (ROC) curves. Thereby, a large number of patients with a predefined clinical outcome, like major adverse cardiovascular events (MACE), are needed. A consensus paper summarised cut-off values for the various laboratory methods and the association of these values with MACE (36).

Prevalence of influencing factors

Published analyses on HRPR are restricted to a rather small group of different investigators, who applied the same method(s) in most of their studies to identify in different or overlapping patients’ populations various aspects associated with HRPR. Within these
patients’ cohorts, they also described the same cofounders of HRPR, which were therefore published repeatedly in several reports. This may lead to an overestimation of the cofounding condition and of the sensitivity of a specific test system for certain influencing variables.

Laboratory evaluation of HRPR

A comprehensive review to evaluate platelet function in the laboratory has been published (37). Therefore, only brief remarks pertinent to the evaluation of HRPR are given to elucidate pros and cons of the most frequently used test systems.

Light transmission aggregometry (LTA)

LTA is not standardised for the estimation of HRPR and thus there is a variability of the applied agonists, and their concentrations, the use of adjusted or unadjusted platelet counts, recording of maximal or final aggregations, and definition cut-off values (8).

Multiple electrode impedance aggregometry (MEA)

This method is relatively good standardised and data from different laboratories can be compared to each other, as long as the manufacturer’s recommendations a strictly followed (38). MEA is the second most frequent method used for the assessment of HRPR.

Vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay

The concept of this assay allows the most direct estimation of P2Y12 ADP receptor inhibition (39). Data from different institutions can be compared to each other, as a commercial kit is available for this purpose.

VerifyNow P2Y12 assay

Data generated with this point-of-care (POC) device are comparable from one laboratory to the next.

Platelet function analyzer-100 (PFA-100)

New cartridges (Innovance PFA P2Y12) were specifically designed to determine HRPR during thienopyridine therapy. Data with these new cartridges were not associated with adverse events (9).

Cone and Plate(let) Analyzer Impact-R

The procedure is not standardised and reproducibility of data is strongly dependent on the operator. The few data generated with this method, are controversial (9, 14, 40).

Association of HRPR with clinical conditions or drugs

Gender predominance

Studies associating gender with ADP-inducible platelet reactivity by only one platelet function test are referenced in Table 1 (12, 18, 41–44).

Seven studies indicate that gender plays a role in HRPR. However, whether male or female patients are more frequently affected remains unclear. By LTA (41), and the VerifyNow P2Y12 assay (12, 44) female patients were more often affected, while in other studies using LTA (18), MEA (19, 42) and impedance aggregometry (43) male individuals had more often HRPR. Of interest, no gender association with the risk of MACE was observed in four studies using the VerifyNow P2Y12 assay (45), LTA and Impact-R concomitantly (14), VASP only (15), or LTA only (10). Thus, gender effects remain controversial.

Advanced age

An association of advanced age with HRPR was identified in 14 publications. Thereby, a clear age cut-off cannot be determined, as aging and thus influence of age on platelet reactivity is a continuous process. One study concomitantly performing LTA and the VerifyNow P2Y12 assay discloses age as a cofounder of HRPR (46). In one study the PFA-100, but not LTA (47), and in two other studies data from LTA but not from flow cytometric measurements indicate this association (48, 49). Single assays were also run to show an association between age and HRPR (Table 1) (11, 12, 18, 50–56). Advanced age comprises many variables, like increased CRP levels, decrease of renal function, and other co-morbidities that may become significant if combined into one single parameter, namely advanced age. Advanced age can therefore be seen as a surrogate marker for the risk of HRPR. At the same time, however, elder patients are at risk for bleedings when treated with potent ADP receptor inhibitors (57).

Body mass index (BMI)

As expected diabetes and increased BMI show a strong overlapping risk for HRPR, but the definition of high BMI varied between different publications (Table 1) (16, 18, 42, 50, 52, 53, 56, 58–63). Only three papers describe the application of more than one assay concomitantly in the same cohorts (19, 48, 49). LTA, MEA and
VASP were all sensitive to high BMI (48, 49), as was concomitant use of MEA and VASP (19).

**Diabetes**

Among all clinical conditions, diabetes is the most frequently identified cofounder of HRPR. LTA was the most common method associating diabetes with HRPR, either as a single method (Table 1) (11, 12, 16, 41, 43, 44, 50–52, 54–56, 59, 60, 62–66) or in combination with other assays, like PFA-100 (47, 67), or flow cytometry, PFA-100, and P2Y12 VerifyNow P2Y12 assay (68). Investigations with LTA, but not flow cytometry investigations revealed diabetes as a cofounder of HRPR (49). MEA was the second most common assay showing diabetes associated with HRPR, either as a single used method (Table 1) (16, 43, 59, 60, 62), or applied concomitantly with the VASP assay (19). In this paper, the latter failed to show such an association, while it did in another publication, when only VASP was investigated (63). There is good evidence that in vitro assays, be it LTA, MEA, or the VerifyNow P2Y12 assay, identify diabetic patients to respond poorly to clopidogrel, and are therefore at an increased risk for MACE. The controversial findings with the VASP assay elucidate that the mechanisms leading to HRPR are manifold. On the one hand, decreased metabolism of clopidogrel and thus provision of its active metabolite may be a consequence of the generally impaired metabolism in diabetes (69). These findings are supported by decreased P2Y12 ADP receptor inhibition as specifically determined by the VASP assay. Moreover, alterations of the platelet membrane due to hyperglycaemia and failure of insulin to inhibit platelet signalling through P2Y12 may be responsible for increased reactivity to ADP (70, 71).

**Impaired renal function**

Impaired renal function was specifically evaluated for its association with HRPR in 10 studies. Concomitantly run assays affirming HRPR in patients with renal failure were LTA and flow cytometry (72), while VASP but not MEA, and the VerifyNow P2Y12 assay but not LTA showed such an association in two other studies (19, 73). The single assays LTA and MEA identified such an association in seven papers (Table 1) (43, 51, 54, 55, 56, 65). Renal function impairment has to be seen as co-morbidity in diabetes, advanced age, and impaired left ventricular ejection fraction (LVEF) which all by themselves contribute to HRPR.

**Left ventricular ejection fraction (LVEF)**

An association of HRPR with left ventricular function is mentioned in 11 studies. Interestingly, one report associated high ejection fraction with HRPR, as estimated by both, PFA-100 and LTA (47). In other reports, using single assays, impaired left ventricular function was associated with HRPR (Table 1) (12, 16, 18, 50, 51, 54–56, 59, 65). These data may be interpreted as the more sick, the worse the response to therapy. Further, impaired LVEF may have a

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**Table 1:** Significant associations of influencing variables with HRPR if evaluated with only one method.

<table>
<thead>
<tr>
<th>Variable</th>
<th>LTA</th>
<th>MEA</th>
<th>VerifyNow</th>
<th>VASP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (f/m)</td>
<td>18,41</td>
<td>42,43</td>
<td>12,44</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>18,50,51</td>
<td>43,44</td>
<td>11,12</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>18,50,52</td>
<td>16,42,59</td>
<td>60,61,62</td>
<td>12</td>
</tr>
<tr>
<td>Diabetes</td>
<td>18,41,50</td>
<td>16,43,59</td>
<td>60,62</td>
<td>11,12,44</td>
</tr>
<tr>
<td>Renal failure</td>
<td>51,54,55</td>
<td>43,60,61</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>LVEF</td>
<td>18,50,51</td>
<td>16,59</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>56</td>
<td>62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet counts</td>
<td>16,59,60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetics</td>
<td>52,53,55,85</td>
<td>42</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>CYP2C19*2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C19*17</td>
<td>52,53,55,85</td>
<td>42</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>PPIs</td>
<td>50</td>
<td>42,60,61,62</td>
<td>44</td>
<td>88</td>
</tr>
<tr>
<td>CCBs</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1HRPR in female/male patients; 2Impedance aggregometry other than MEA; 3smoking was associated with HRPR, while an inverse association was reported in all other studies; 4increased platelet inhibition in carriers; LTA, light transmission aggregometry; MEA, multiple electrode aggregometry; VASP, vasodilator-stimulated phosphoprotein; BMI, body mass index; LVEF, left ventricular ejection fraction; PPIs, proton pump inhibitors; CCBs, calcium-channel blockers.
detrimental effect on renal function, liver metabolism and other mechanisms that are involved in absorption and metabolism of clopidogrel.

**Inflammation (based on C-reactive protein (CRP) levels and/or leukocyte counts)**

In one study using LTA and PFA-100 inflammation was associated with HRPR (47). Further, both MEA and VASP data were associated with HRPR in inflammation (19). Likewise, MEA and LTA as the only assay, respectively, were associated with inflammation (Table 1) (56, 62). Interestingly, one study reports lower platelet reactivity assessed by the VerifyNow P2Y12 assay in patients with increased levels of CRP (46). An interpretation of these data needs to be seen with those in patients with high platelet counts. However, only one report, which indicated an association of high platelet counts with HRPR found such an association also with inflammation (19).

**Platelet count**

Most assays require a platelet count of 80 to 100 G/l, otherwise the data indicate low platelet function. Little is known about the reactivity if platelets are close to the upper range seen in otherwise healthy individuals, or even at platelet counts above the normal range. MEA seems particularly sensitive to high platelet counts, as these were described to be associated with HRPR in three studies (Table 1) (16, 59, 60). Interestingly, in one study by MEA (but not by the VASP assay) high platelet counts were associated with a good response to clopidogrel (19). It is clear that whole, undiluted blood with high platelet counts may result in high residual platelet reactivity in the MEA assay, while this is not seen in other assays like LTA or flow cytometry, when the platelet count is adjusted. However, other assays using undiluted blood like the VerifyNow P2Y12 assay, Impact-R, and the PFA-100 are also expected to be sensitive not only to low, but also high platelet counts. No significant association between platelet counts and HRPR was reported if these assays were used. High platelet counts result often from an increased levels of CRP (46). An interpretation of these data needs to be seen with those in patients with high platelet counts. However, only one report, which indicated an association of high platelet counts with HRPR found such an association also with inflammation (19).

**Smoking**

Most investigations show better inhibition of ADP-inducible platelet aggregation in current smokers than in non-smokers. This was explained by the induction of cytochrome P450 enzymes needed to metabolise clopidogrel to its active P2Y12 inhibitor. The opposite is described in several studies from the same group of investigators using MEA (16, 59–62). As shown in Table 1, the VerifyNow P2Y12 assay was the assay showing an association of smoking with good response to clopidogrel, whereas smoking was associated with HRPR by MEA (12, 16, 44, 59–62, 65, 74). Furthermore, in two other studies applying the VerifyNow P2Y12 assay together with other methods, only the VerifyNow P2Y12 assay was sensitive to smoking (46, 73). The beneficial effect of smoking on platelet responsiveness to ADP was also demonstrated in one study using LTA and flow cytometric evaluation of glycoprotein IIb/IIIa expression (75). Apparently, it is difficult to verify the hypothesis that smoking induces clopidogrel metabolism, as such a report has not been published so far. Further, it would be interesting to specifically address the association of a good response in smoking versus non-smoking individuals in association with the genetic predispositions to metabolise clopidogrel.

**Genetics**

Various genetic predispositions were linked to HRPR and MACE, respectively (7, 49, 53, 76–84). The most common association of HRPR was seen with the loss of function allele CYP2C19*2, be it by single method analyses (Table 1) (42, 52, 53, 55, 63, 85), or be it in combined analyses, like LTA and flow cytometry (49), or LTA together with the VASP assay and flow cytometry (86). Activation of glycoprotein IIb/IIIa was also associated with genetic variants (87). The CYP2C19*2 genotype accounted for only 12% of the variability in clopidogrel response (53). However, meta-analyses clearly show an association of CYP2C19*2 with MACE (7, 76, 77), As a consequence, the U.S. Food and Drug Administration approved a new label for clopidogrel with a “boxed warning” on the diminished effectiveness of the drug in patients with impaired ability to convert clopidogrel to its active form. This “boxed warning” serves to make clinicians aware of the imperfect, but significant, knowledge that we have on response to clopidogrel in case of genetic variations and to emphasise that clinicians should use this knowledge to make decisions about how to treat individual patients. Whether or not genotyping data are complementary to platelet function testing is unknown. It also remains unclear if these genetic predispositions affect the metabolism of substances that are not artificial drugs, like clopidogrel, and are therefore evolutionary advantageous or not.

**Proton pump inhibitors (PPIs)**

Among all drugs that are concomitantly given to patients with atherosclerosis PPIs have been investigated most extensively for their interaction with clopidogrel metabolism. Various PPIs are used to protect from gastrointestinal bleeding, in particular in patients with concomitant aspirin therapy. Of note, PPIs are more frequently given to older patients, thus a patient cohort that has been associated with HRPR (46, 51). Some PPIs may compete with clopidogrel for CYP2C19 of the cytochrome P450 enzyme system and thereby attenuate the metabolism of clopidogrel to its active P2Y12 inhibitor. Single methods disclosing an effect of PPIs on clopidogrel meditated-platelet inhibition are shown in Table 1 (42, 44, 44, 50, 60–62, 88). Among multiple methods analyses,

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MEA and VASP revealed concordant data (19), while VASP, but not LTA disclosed a PPI effect (89). If different PPIs were compared to each other, an omparable effect was seen (60). A combined application of LTA and the VASP assay and of LTA, the VASP assay, the VerifyNow P2Y12 assay, Impact R, and MEA did not reveal an association of PPIs with HRPR (90, 91). Of note, in a recent meta-analysis and in a large cohort study the use of PPIs was not associated with an increased risk for MACE, underlining the need of cautious interpretation of in vitro testing for HRPR (92, 93).

Calcium-channel blockers (CCBs)

Seven papers addressed an association of CCBs with HRPR, with the rationale that these drugs may compete with clopidogrel for CYP3A4 of the cytochrome P450 enzyme system needed to metabolise clopidogrel to its active P2Y12 inhibitor. LTA was the most common assay to show such an association of CCBs with HRPR. Three studies reported congruent LTA data with the VerifyNow P2Y12 assay (46, 73, 94), and one between MEA and the VASP assay (95). One group revealed an association of CCBs with HRPR by the VASP assay but not MEA (19), and one study revealed an association of concomitant CCB medication with HRPR by LTA but not by flow cytometric data (49). One study evaluated the effect of CCBs by only one test system (Table 1) (51).

Statins

Several studies addressed the influence of lipophilic statins on HRPR with the rationale that these statins may compete with clopidogrel for CYP3A4 of the cytochrome P450 enzyme system. Three studies found no association with LTA and flow cytometry (96, 97), or with LTA as a single test (54). One group described an association between statin medication and HRPR when assessed by LTA (Table 1) (50). In conclusion, current data are not sufficient to show an association of HRPR in patients treated with statins.

Conclusion

There is a dynamic response to clopidogrel that is based on clinical conditions of patients, like co-morbidities, genetic predispositions, and along with the latter, interactions with other drugs. It is apparent that the rate of response to clopidogrel depends on the interval of the in vitro investigation from loading and disease progression, respectively (8). This dynamic response may further determine whether or not any of the different variables indeed affects residual ADP inducible platelet activation. Therefore, results are heterogeneous, with just few exemptions. It shall also be noted that although HRPR indeed suffer from adverse events. These observations further suggest that in vitro HRPR may not be suitable to guide therapy in each patient. There are no data so far, showing that adjustment of therapy based on in vitro testing is advantageous for the patient (98). If therapy was adjusted based on in vitro evaluation of platelet response, a large proportion of patients would be treated with a regimen that they do not need, may render these individuals to a risk of bleedings.

Conflict of interest

None declared.

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